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Reduction in channel catfish hepatic growth hormone receptor expression in response to food deprivation and exogenous cortisol

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Abstract

The objective of this study was to assess the effects of food deprivation and exogenous cortisol administration on somatic growth of channel catfish, *Ictalurus punctatus*, and examine the resultant changes in circulating insulin-like growth factor-I (IGF-I) concentrations and growth hormone receptor (GHR) gene expression. Integral to this objective, we report the isolation, sequence, and characterization of channel catfish GHR. Sequence analysis and characterization results indicate sequence identity and tissue distribution similar to GHRs in other teleost fish and several functional characteristics conserved in known vertebrate GHRs. The effects of food deprivation and dietary exogenous cortisol administration were assessed as part of a 4-week study. Growth was significantly reduced after 4 weeks in cortisol-fed fish compared to fed-control fish, and fasting resulted in weight loss. At the end of the 4-week study, both IGF-I plasma concentrations and hepatic GHR mRNA abundance were significantly reduced in fasted and cortisol-fed catfish. Levels of hepatic GHR mRNA were positively correlated to circulating IGF-I levels. These results suggest that a reduction in hepatic GHR gene expression might serve as a mechanism for the reduction of circulating IGF-I and growth in channel catfish during periods of food deprivation and stress.
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Keywords: Channel catfish growth hormone receptor; Sequence; Expression; Fasting; Cortisol

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1. Introduction

Channel catfish, *Ictalurus punctatus*, farming is by volume the largest aquaculture enterprise in the United States. Periods of stress and fasting are inherent to catfish culture as a result of management, disease, and environmental conditions. While it is widely accepted that both fasting and stress can reduce growth, the pathways through which growth is affected are not clearly defined in catfish, especially where stress is involved. Catfish, like other teleost fishes, present elevated levels of plasma cortisol following acute and chronic stress. Increased circulating cortisol levels have been linked to changes in energy metabolism [1,2] and reduced fish growth [3]. Furthermore, exogenous cortisol administration has been shown to reduce growth in both rainbow trout, *Oncorhynchus mykiss* [4] and channel catfish [5], thus linking elevated plasma cortisol levels and stress-related changes in growth.

Cortisol is present at low but detectable levels even in quiescent fish, suggesting it is important for normal growth, and some level of cortisol response to stress, whether physiological (such as food deprivation) or perceived stress, may be adaptive. Cortisol is a multifaceted hormone in fish and plays important roles in intermediary metabolism [6–8], ionic and osmotic regulation [9], and immune function [10], supporting the argument for an adaptive role. Although a clear relationship between fasting and cortisol has not been established for channel catfish, fluctuations in plasma cortisol concentrations in response to fasting suggest the possibility of a dynamic response to metabolic status in these animals [11,12]. Similar observations reported for other fish species suggest a possible bi-directional relationship between feed intake and circulating glucocorticoid levels [13].

Knowledge of cortisol's action on the somatotropic axis in fish is limited. Only a few studies have reported the effects of stress or exogenous cortisol on growth and somatotropic hormones in fish [14–18]. Among those, Peterson and Small [18] found that feeding exogenous cortisol to channel catfish reduced weight gain and plasma insulin-like growth factor-I (IGF-I) levels. At the highest level of cortisol administration, Peterson and Small [18] also observed increased pituitary growth hormone (GH) mRNA abundance. These observations, decreased IGF-I and increased GH, are similar to those reported following long-term fasting in channel catfish [19].

Growth hormone is generally accepted to play a central role in the regulation of somatic growth. As in other vertebrates, the actions of GH in fish are mediated by IGF-I, primarily produced in the liver [20]. The actions of GH on target tissues, such as the liver, and IGF-I are initiated by interactions with membrane-bound GH receptor (GHR). The structure of GHR has been elucidated through cDNA cloning in many vertebrate species, mostly mammalian [21]. More recently, since the cloning and sequencing of GHRs in turbot, *Scophthalmus maximus* [22] and goldfish, *Carassius auratus* [23], a small number of other fish GHRs have also been sequenced and characterized [24].

The mechanisms through which fasting and cortisol alter growth and IGF-I levels in fish are largely unknown. During fasting, GHR expression is down-regulated in many vertebrates, resulting in a decrease in circulating IGF-I and an increase in GH [25]. Although the functional characterization of GHR genes in fishes has only recently begun, Fukada et al. [26] have reported decreased GHR expression in the liver of fasted masu salmon,

Oncorhynchus masou. Observed similarities in GH and IGF-I responses of fasted [11,19] and cortisol-fed [18] channel catfish suggest that similar mechanisms, involving the down-regulation of GHR, might be involved. To better understand the regulation of channel catfish growth during fasting and periods of elevated plasma cortisol, the channel catfish GHR gene was cloned and characterized for the development of a real-time PCR assay to measure gene expression. It was hypothesized that fasting and elevated circulating cortisol concentrations, as separate physiological processes, would result in a reduction of hepatic GHR expression. Here we report the sequence and characterization of channel catfish GHR and changes in hepatic GHR mRNA levels of fed, food-deprived, and cortisol-fed catfish in relation to growth and plasma IGF-I levels after 4 weeks of respective treatments.

2. Materials and methods

2.1. Channel catfish GHR isolation, analysis, and characterization

2.1.1. Isolation and sequencing

Two primers were designed from a 907 bp blue catfish (*Ictalurus furcatus*) and a 662 bp channel catfish expressed sequence tag (EST) generated from liver cDNA libraries and annotated as similar to growth hormone receptor (GenBank Accession Nos. CK406496 and BM438611, respectively). The two primers, GHR-X2-F2 (5′-AGACCCACCTGAAGTGCTGAAC-3′) and GHR-X2-R2 (5′-TAGCCATGAGGATAA-ACACTGC-3′), were used to screen a channel catfish BAC library by PCR as previously described [27]. Briefly, the 15 μL reaction contained 1.5 μL of 10× buffer (600 mM Tris–HCl (pH 9.25), 15 mM $MgSO_4$, 300 mM NaCl, 0.1 mg/mL BSA, 0.1% Triton X-100), 800 nM of each primer, 200 nM of dNTP, 2 ng (1 μL) of DNA template, 0.5 units of ThermalAce DNA polymerase (Invitrogen, Carlsbad, CA). The PCR parameters were 95 °C for 2 min, then 30 cycles of 95 °C for 60 s, 60 °C for 30 s, 72 °C for 60 s, then a final extension of 72 °C for 10 min. Two positive clones (CCBL1_38B01 and CCBL1_11L09) were identified and directly sequenced by primer walking using ABI PRISM BigDye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 DNA Analyzer.

Transcriptional boundaries were determined by rapid amplification of cDNA ends (RACE). The BD SMART RACE cDNA amplification kit (BD Biosciences, San Jose, CA) was used for amplification of the 3′ untranslated region of channel catfish GHR. Total RNA isolated from channel catfish liver tissue was used to produce 3′ RACE cDNA according to the manufacturer's protocol. One microliter of the 3′ RACE cDNA served as template in the first round of nested PCR, along with 1× PCR buffer, 1.25 units of Taq polymerase, 200 nM dNTPs, 1 mM $MgCl_2$, and 0.8 μM of each primer in a final reaction volume of 25 μL. Specifically, the gene specific primer GHR-BAC-F3 (5′-ATTCTTCTGCCACCTGTTCC-3′) was used in combination with the 10× Universal Primer Mix (supplied by the manufacturer) in this first round of PCR. The PCR parameters were 35 cycles of 94 °C for 15 s, 60 °C for 20 s, 72 °C for 120 s, and a final extension of 72 °C for 8 min. For the second round of nested PCR, 0.5 μL of a 1/20 dilution of the first round PCR was used as template, along with the

primers GHR-BAC-F4 (5′-ACCCCATTGCACAACCAGAG-3′) and the Nested Universal Primer Mix (supplied by the manufacturer). The PCR conditions and cycling parameters were identical to those listed for the first round of 3′ RACE PCR.

Ambion FirstChoice RNA ligase-mediated 5′ RACE kit (Ambion Inc., Austin, TX) was used for the amplification of the 5′ untranslated region of GHR. Total RNA from channel catfish liver was prepared according to the manufacturer's protocol. The prepared RNA sample was used for first-strand 5′ RACE cDNA synthesis. The 5′ RACE cDNA (0.5 μL) was used as template in the first round of nested PCR, in combination with 1× PCR buffer, 1.25 units of Taq polymerase, 200 nM dNTPs, 1 mM $MgCl_2$, and 0.4 μM of each primer in a final reaction volume of 25 μL. The reaction cycling parameters were 35 cycles of 94 °C for 15 s, 60 °C for 20 s, 72 °C for 120 s, and a final extension of 72 °C for 8 min. Primers GHR-X3-R1 (5′-TGCCATGCAGGTTCCTGTG-3′) and 5′ RACE Outer Primer (supplied by the manufacturer) were used in the first round of PCR. The second round of nested PCR utilized 0.5 μL of the first round PCR as template, along with the primers GHR-BAC-R3 (5′-GGGTCATTACTATAGAGCTCTGG-3′) and the 5′ RACE Inner Primer (supplied by the manufacturer). The PCR conditions and cycling parameters for the second round of PCR were identical to those listed for the first round of 5′ RACE PCR.

Oligonucleotide primers GHR-5p-F2 (5′-CGGATGCGGGATTAATGAGAG-3′) and GHR-3p-R1 (5-AAACAAGCCACGCAGAGAGTC-3′) were used for the amplification of a single cDNA fragment that included the entire open reading frame of channel catfish GHR. This PCR was performed using a proof-reading DNA polymerase (Invitrogen). Specifically, the reaction conditions were as follows: 1× Accuprime Pfx reaction mix, 0.5 μM of each primer, 0.2 μL liver cDNA, and 0.5 units of Accuprime Pfx DNA polymerase in a final reaction volume of 20 μL. The reaction cycling parameters were 35 cycles of 95 °C for 15 s, 55 °C for 30 s, and 68 °C for 150 s, and a final extension for 15 min at 72 °C with 0.5 units of Taq polymerase to facilitate the addition of A-overhangs to the 3′ ends of the amplicon so that the fragment could be easily cloned into the pCR4-TOPO vector (Invitrogen) for sequencing.

Amplification products were cloned into pCR4-TOPO vector (Invitrogen), and putative GHR recombinants were identified by PCR screening. Positive recombinants were grown overnight in 5 mL of LB/ampicillin (100 μg/mL) at 37 °C. Recombinant plasmids were purified using a standard alkaline lysis protocol. Plasmid DNA sequencing reactions were conducted using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequencing was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

2.1.2. Sequence analysis and characterization

DNA and translated protein sequence homologies were identified using BLASTN, BLASTX, and BLASTP (http://www.ncbi.nlm.nih.gov). Significant identities were assumed at a level of $P < 0.0001$. Multiple alignments of cDNA and amino acid sequences were performed using CLUSTALW (http://workbench.sdsc.edu). Prediction of functional sites was performed using the eukaryotic linear motif (ELM) resource of functional sites in proteins (http://elm.eu.org). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 [28].

Table 1
Nucleotide sequences of PCR primers and probes used to assay gene expression by real-time PCR

Gene	Primer	Sequence
GHR	Sense	CTGTGAGCTGAAACGACTCG
	Anti-sense	CCAGATATAGGTTTTCTTTGGTG
	Probe[a]	CGAGAGCAACGGCACACAGACACGC
18S	Sense	GAGAAACGGCTACCACATCC
	Anti-sense	GATACGCTCATTCCGATTACAG
	Probe[b]	GGTAATTTGCGCGCCTGCTGCC

[a] The probe for GHR (GenBank Accession No. DQ103502) were dual-labeled with a reporter dye (FAM, 6-carboxyfluorescein) at the 5′ end and a quencher dye (Black Hole quencher-1, BHQ-1) at the 3′ end (Biosearch Technologies, Novato, CA).

[b] The probe for α-tubulin (GenBank Accession No. AF021880) was dual-labeled with a reporter dye (HEX, hexachlorofluorescein) at the 5′ end and a quencher dye (BHQ-1) at the 3′ end (Biosearch Technologies).

2.1.3. Gene expression and tissue distribution

Total RNA of the hypothalamus, pituitary, gill, muscle, heart, liver, spleen, trunk kidney, head kidney, pancreas, stomach, gall bladder, and intestinal tracts was extracted separately from four ∼100 g fish using TRI-reagent (Molecular Research Center, Cincinatti, OH) according to the manufacturer's protocol. Total RNA was quantified on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE), and 1 μg of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's instructions. The synthesized cDNA was then quantified and diluted to 200 ng/mL for all samples. A total of 400 ng of cDNA was used in expression analyses. Gene expression was determined by real-time quantitative PCR using the iCycler iQ^{TM} Real Time PCR Detection System (Bio-Rad Laboratories) to measure mRNA abundance. Standards were developed by generating PCR fragments using the primers listed in Table 1, and then cloning them into the pCR4-TOPO vector (Invitrogen). Cloned inserts were sequenced to confirm sequence identity. Concentration of each resulting plasmid was measured spectrophotometrically, and serial dilutions of each plasmid were used to make the standard curves for quantification. Primer and probe sequences for the target genes are listed in Table 1. The standard curve showed a linear relationship between cycle threshold values and the logarithm of input gene copy number. All real-time PCR amplifications were performed in triplicate, and specific quantities were normalized as copy number against total RNA to avoid standardize concentrations across tissues (Bustin, 2002). Each PCR mixture (12.5 μL) contained 400 ng of cDNA; 1× iQ Supermix (Bio-Rad Laboratories) which consisted of: 10 mM KCl, 4 mM Tris–HCl, pH 8.4, 0.16 mM dNTPs, 5 U/mL iTaq polymerase, 0.6 mM $MgCl_2$ and stabilizers; 10 nM of each primer; and 20 nM dual-labeled probe. The amplification profile was 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 55 °C for 60 s. Amplification products were quantified by comparison of experimental Ct (threshold cycle—defined as the PCR cycle where an increase in fluorescence over background levels first occurred) levels with those of the standard curve. The standard curve for each gene was generated from two replicates of serial dilutions of recombinant plasmid.

2.2. Feeding study

2.2.1. Animals and experimental design

Channel catfish used in this study were from the NWAC103 strain. Catfish (30.6 ± 3.0 g) were randomly stocked into nine, 76-L aquaria (seven fish/tank) and allowed to acclimate for 2 weeks, then randomly assigned to one of three treatments: (1) fed-control, (2) fasted (food deprived), and (3) cortisol-fed (dietary cortisol provided at 200 mg/kg feed). Feed was offered to apparent satiation once daily in the morning for 4 weeks. Apparent satiation was achieved by offering small quantities of feed to the fish by hand until feeding activity stopped. Cortisol-laden feed was prepared by dissolving the appropriate amount of cortisol (Sigma–Aldrich, St. Louis, MO) in ethanol and spraying it on a floating commercial fish feed containing 36% crude protein (Land O Lakes Farmland Feed, Arden Hills, MN). At the end of the study, all the fish were anesthetized as described below and weighed, then two fish per tank were euthanized, bled, and their livers excised. Throughout the study, the fish were reared in 26.0 °C flow-through well water and maintained with a 14-h light:10-h dark photoperiod. Water quality (pH 8.5 and dissolved oxygen levels > 5.0 mg/L) and flow rates were similar between treatments.

2.2.2. Blood collection and tissue preparation

Whole blood was collected from the caudal vasculature into syringes coated with heparin. Fish were initially anesthetized with 0.6 mg/L metomidate hydrochloride prior to blood collection, then euthanized in a 200 mg/L solution of tricaine methanesulphonate (TMS, MS-222) prior to dissection. Plasma was separated, stored at −80 °C, and later analyzed for IGF-I. Hepatic tissue specimens (∼100 mg) for total RNA extraction were rapidly excised from each fish, immediately placed in 1 mL TRIzol (Life Technologies), flash-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation.

2.2.3. Plasma IGF-I analysis

Plasma IGF-I concentrations were determined using a heterologous time-resolved fluoroimmunoassay (TR-FIA) validated for quantifying circulating levels of channel catfish IGF-I [19]. Sensitivity of the assay was 0.20 ng/mL, and recovery of IGF-I from spiked plasma samples was ≥95%. Intra- and inter-assay CV's were 5.1 and 9.8%, respectively. Dose–response inhibition curves using serially-diluted plasma samples consistently showed parallelism with the IGF-I standard curve.

2.2.4. RNA isolation and hepatic GHR expression

A 100 mg section of liver was excised for RNA extraction. Samples were immediately placed in 1 mL TRIzol and flash-frozen in liquid nitrogen, and stored at −80 °C until RNA isolation, as described above. The integrity of the RNA preparations was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 2.0% agarose gels. GHR gene expression was determined by real-time PCR as described above. All specific quantities were normalized against 18S ribosomal RNA levels. 18S ribosomal RNA levels were not affected by fasting. Primer and probe sequences for the target genes are listed in Table 1.

2.3. Statistical analyses

The experimental data for all treatments (fed, food-deprived, and cortisol-fed) were subjected to analysis of variance (ANOVA) mixed-model procedures using SAS software system version 8.00 (SAS Institute, Cary, NC), with treatment group as a fixed effect and aquarium within treatment group as a random effect. For all analyses, aquarium was the experimental unit and aquarium within treatment group mean square was used as the error term in tests of differences among treatment group means. When significant differences were found using ANOVA, pairwise contrasts were made using an LSD test to identify significant differences at $P < 0.05$. Results are presented as mean + pooled standard error (S.E.).

3. Results

3.1. Channel catfish GHR DNA isolation and characterization

3.1.1. GHR sequence

PCR screening of the channel catfish BAC library yielded two positive clones for GHR. Subsequently, catfish GHR genomic DNA was sequenced from one of the positive clones containing the entire GHR gene (Fig. 1). The genomic DNA consisted of six exons and five introns. The second intron contained an AT repeat of more than 240 bp which prevented sequencing the entire intron. The GHR cDNA was sequenced from RACE products and PCR amplification of hepatic cDNA, and was 2795 bp in length. The channel catfish GHR cDNA sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank DNA database under Accession No. DQ103502, and genomic DNA sequences deposited under Accession Nos. DQ241381 and DQ241382.

3.1.2. Sequence analysis and characterization

The GHR coding region consisted of 1665 bp and encoded 556 amino acids (aa) containing a putative 20 aa signal peptide, a 221 aa extracellular domain, a 23 aa transmembrane domain, and a 291 aa intracellular domain. Phylogenetic analysis demonstrated conservation of the GHR coding region, with the channel catfish receptor being most similar to salmonid GHRs (Fig. 2). Multiple alignment of amino acid sequences revealed that channel catfish GHR had the highest sequence homology (46%) to Coho salmon, *Oncorhynchus kisutch* (GenPept Accession No. AAK95624) GHR. Channel catfish GHR also shares significant sequence similarity with Atlantic salmon, *Salmo salar* (AAS17950) (45%), Japanese eel, *Anguilla japonica* (BAD20706) (41%), common carp, *Cyprinus carpio* (AAT92555) (39%), and turbot, *Scophthalmus maximus* (AAK72952) (36%), chicken, *Gallus gallus* (AAA48781) (33%), and human, *Homo sapiens* (NP_000154) (32%) GHR. When compared to rainbow trout, *Oncorhynchus mykiss* prolactin receptor (AAG44267), channel catfish GHR shares only 21% similarity.

Within the deduced amino acid sequence of channel catfish GHR, several characteristics conserved in known vertebrate GHRs were identified. These include conserved

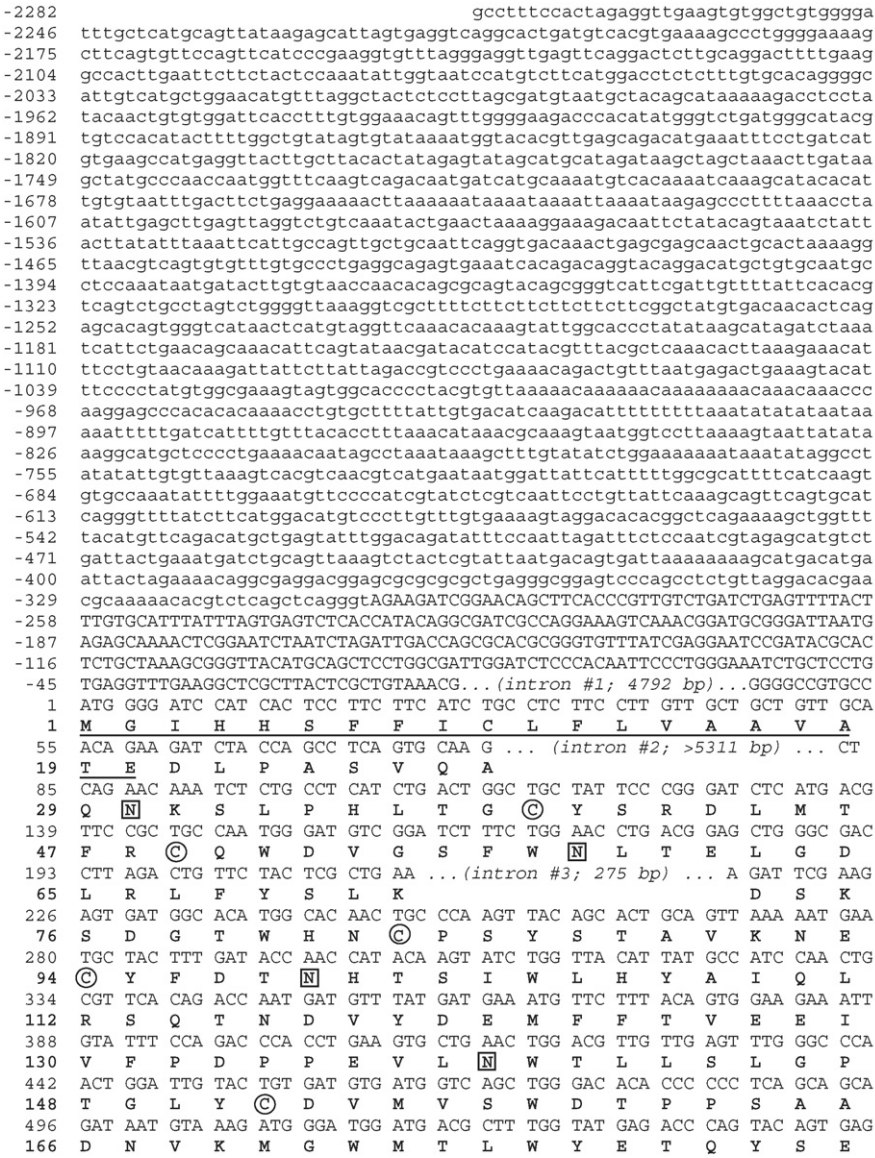

Fig. 1. Genomic DNA and deduced amino acid sequences of channel catfish growth hormone receptor. Capitalized nucleotides denote the corresponding cDNA sequence (GenBank Accession No. DQ103502). The putatve signal peptide is single underlined. Conserved cysteine residues are denoted by a circle (◯), potential *N*-linked glycosylation sites are denoted by a square (□), and conserved tyrosine residues are denoted by a diamond (◊) around the amino acid. The transmembrane domain is double underlined, Box 1 and Box 2 regions are boxed in shaded rectangles, the stop codon is denoted by an asterisk, and the polyadenylation signal is in bold letters at position 2442.

```
 550  AGG GGC TCA GAG CAG TGG AAA TCT ... (intron #4; 640 bp) ... CTC GAC AGT
 184  R   G   S   E   Q   W   K   S                                L   D   S
 583  GGC AAG GAC ACA CAG GCA AAT ATC TAC GGC CTT CGC AGC AAC ACT GAG TAT GAA
 195  G   K   D   T   Q   A   N   I   Y   G   L   R   S   N   T   E   Y   E
 637  GTC AGG GTG AGG TCC AAA ATG AGG GGC TAC AAC TTT GGG GAT TTT GGT GAC TCC
 213  V   R   V   R   S   K   M   R   G   Y   N   F   G   D   F   G   D   S
 691  ATT TTC ATA CTT GTT CCC AGC AAG GGC TCA AGA ATC CCC ATA ACT GCA GTG TTT
 231  I   F   I   L   V   P   S   K   G   S   R   I   P   I   T   A   V   F
 745  ATC CTC ATG GCT ACT GCT ATT GTA ATC ATG TTG ATC CTG ATC GTG GTG TCA CGT
 249  I   L   M   A   T   A   I   V   I   M   L   I   L   I   V   V   S   R
 799  AAA CAG AAA CTC ATG GTG ATT CTT CTG CCA CCT GTT CCT GGA CCA AAA ATC AAA
 267  K   Q   K   L   M   V   I   L   L   P   P   V   P   G   P   K   I   K
 853  GGA ATT GAT CCC CTC CTC CTA CAG ... (intron #5; 1388 bp)... AAG GGC CAG
 285  G   I   D   P   L   L   L   Q                                K   G   Q
 886  CTA ACC GAG TTC ACC TCG GTC CTG GGT GCT CAC CCA GAT CTG CGT CCA GAG CTC
 296  L   T   E   F   T   S   V   L   G   A   H   P   D   L   R   P   E   L
 940  TAT AGT AAT GAC CCA TGG GTG GAG TTC ATA CAG GTG GAC ATT GAC GAA CCG ACT
 314  Y   S   N   D   P   W   V   E   F   I   Q   V   D   I   D   E   P   T
 994  GAG ATG ATT GAA GGC TTG GAC ACA CCA CTC CTC TTT GAC AAA TCT CAT GTC TCT
 332  E   M   I   E   G   L   D   T   P   L   L   F   D   K   S   H   V   S
1048  GAT TCC CCT CCG ACA TCC AGT GGG TTC CAG GAT GAC GAC TCG GGT CGG GCG AGC
 350  D   S   P   P   T   S   S   G   F   Q   D   D   D   S   G   R   A   S
1102  TGC TGT GAC CCC GAT CTG TCT GAT CAT GAC CAC CAA GAT GCT AAT CAC CCT TCA
 368  C   C   D   P   D   L   S   D   H   D   H   Q   D   A   N   H   P   S
1156  ACC AGT GCC CAA GAT TGT TTT CAC ACT TTT ATA CCT CCT GAA AAT TTG GGA TCT
 386  T   S   A   Q   D   C   F   H   T   F   I   P   P   E   N   L   G   S
1210  CTG CAA CCT GTT GTT TCT CCT GCA CAG GAA CCT GCA TGG CAG AAT TCC ATT TAC
 404  L   Q   P   V   V   S   P   A   Q   E   P   A   W   Q   N   S   I   Y
1264  TCT CAA GTG GCT GAA ATT ATG CCA TGT GGT GAA ACA CTG CTG TTC CCA GAG CAG
 422  S   Q   V   A   E   I   M   P   C   G   E   T   L   L   F   P   E   Q
1318  AAC GTG ACT GAT GGC TGC AGC GTT CAA AAT AAA GCT GAG TAT AAA GAG AAA AAT
 440  N   V   T   D   G   C   S   V   Q   N   K   A   E   Y   K   E   K   N
1372  CCC TGG ATG ATG GTG ACC CTT AAT GAA AGA GGT TAC AGT ACC AAC GAG CCA AGC
 458  P   W   M   M   V   T   L   N   E   R   G   Y   S   T   N   E   P   S
1426  TCA TCC ACA GCA AAC CCT GAG TCA GAA AGC AAC AGT ACT CCA CAA CAG TGT CAG
 476  S   S   T   A   N   P   E   S   E   S   N   S   T   P   Q   Q   C   Q
1480  AAC CCC ATT GCA CAA CCA GAG GTG ACA CCC ATC CTC TCT GCC TTT CCT ATC CTC
 494  N   P   I   A   Q   P   E   V   T   P   I   L   S   A   F   P   I   L
1534  ACG ATG CCG AAT CCT CCA GTA TAC ACT ATG GTG GAT GGA GTG GGC TGG AAA GAT
 512  T   M   P   N   P   P   V   Y   T   M   V   D   G   V   G   W   K   D
1588  AGC CTT GTT TTG AAG GCA AGC AAA CAT CCT GGG GGA TAT TTG ACT CCT GAC CTA
 530  S   L   V   L   K   A   S   K   H   P   G   G   Y   L   T   P   D   L
1642  CTA GAC AGC ATT ACT CCA AAT AAG TAG TGCCAGAAATGTACTGAGATGGGATTTGGGGAAAG
 548  L   D   S   I   T   P   N   K   *
1704  AATAGATTAGCTGTAAAGCACGTTGGACATTGTTAGCCAACACCAAACAAGACTGGGCTAAAAACTTGGAA
1775  TCCAAGCAGATTTTTCACTGACTGGTCCTTATTATATTACTGAAACATTTTGGCAAGATGGACTTGGACTG
1846  CTGGAGCTGCTACCCCCGCGACCCGATTACGGATAAGCGGTTGAGGATGGATGGATGGATGGATGGATGGA
1917  TGGACTTGGACTGGATTAGATTTATTGGATGAAATTACAATGACCTATTTTCTGAGCTGTATAAATATTGC
1988  AAATCATAGTTAATTACTGTTTACAACTATAAGGAATGATGCCGTTAAATGGAATTATAGCTGGGTGGATT
2059  AGTATATCTTTTGTATAAAATCTTCTCAGTGGATTAATGAACATTAACATGGATGAGGAACCTGCTCATTT
2130  GTTTGCATTCTCTATCTAACAGCACCGTTTCTAACCCTAGCAAGCATAAGCTAATTTTTGCTGAAGTGCAC
2201  TTGTAAGTCCAGAACTTTTTCTCTAAACCCCAGCACACAATGTATCTGGGTTACACCACAAATGCCTCATC
2272  TTGTTTGTGTTTTTTTTCTATTCTGTTCTGTTCTGCTCTGCAGTGTCTAGCTAGGTAAGTTTCTAAAAAGT
2343  AAGTGTCAATGTCAATAGATATGACTCTCTGCGTGGCTTGTTAAAATGTAATATATATTATAATAAAGAAC
2414  AGATGTATATATTTACAAAAAAGTGAGC AATTAAA GTTTACATTTATACatttctgcttaatcattcgagt
2485  cgattcgtgaattgattcaaagactcaacactttggattcgactctcaattcctaatacacgggaatcgaa
2556  gagccgactcttcgtttgattcatctgccagctgcgattcaaccgtggattttcaagttaaaatgaagaaa
2627  acggttgtcaacctcgataaactaattcaagacttcagtcttctggagcaagtatatcactgacaacacga
2698  tacaaccttgtttgtttgtttgtttttcttcctgagagagattaacaactagtaactgtaaagtctgttag
2769  cgagttagctagctgaggacgccatgtttgattgatttgattgacctgaaagctgtttgtgttcataaaga
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Fig. 1. (*Continued*).

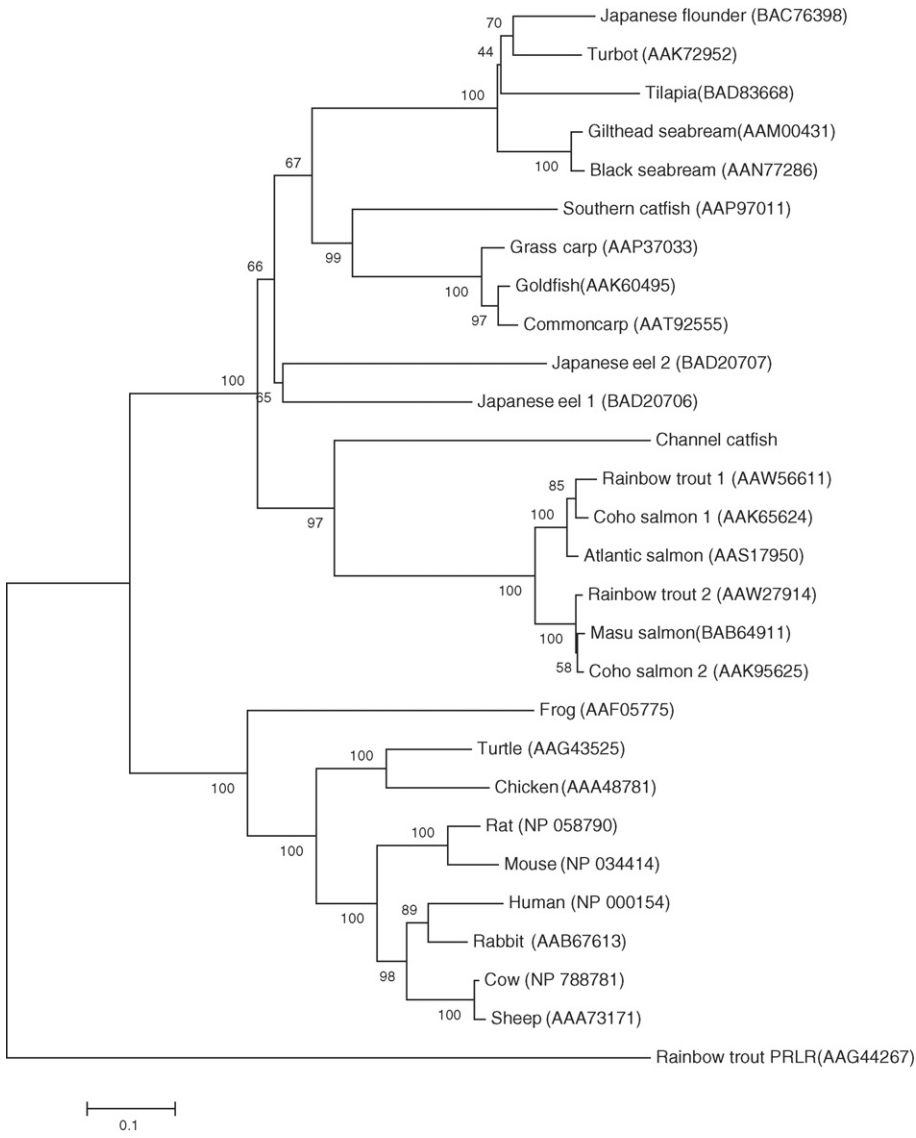

Fig. 2. Phylogenetic tree based on the alignment of amino acid sequences of known vertebrate growth hormone receptors and rainbow trout prolactin receptor (PRLR). The numbers at each node are bootstrap values, and branch lengths indicate proportionality to amino acid changes on the branch. The scale bar shows substitutions per site. GenPept Accession Nos. are presented in parentheses.

cysteine residues, potential *N*-linked glycosylation sites, and a fibronectin type III domain (134–223 bp) in the extracellular domain, a single transmembrane region, and Box 1 and Box 2 regions and conserved tyrosine residues within the intracellular domain (Fig. 1).

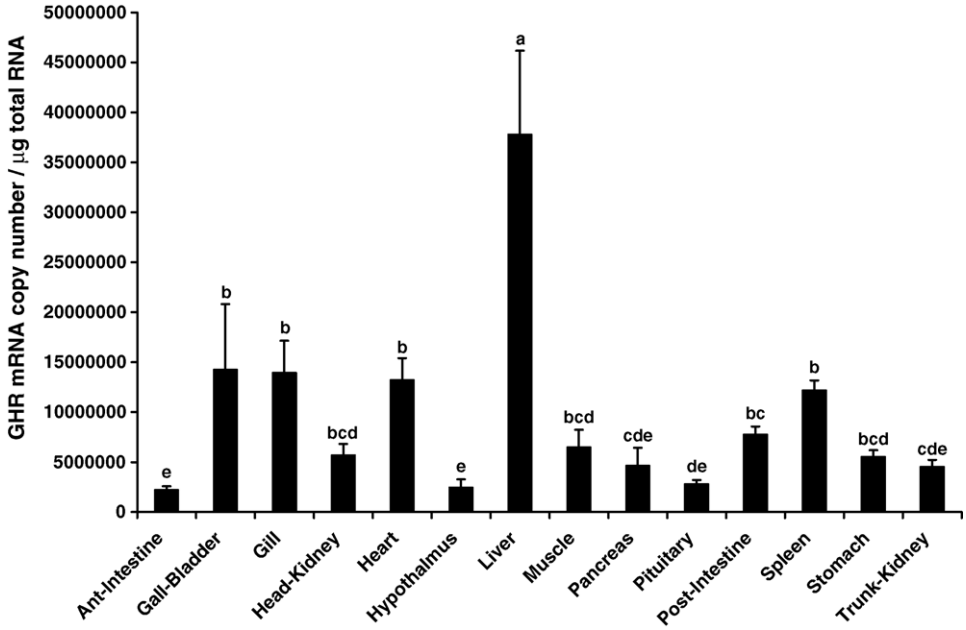

Fig. 3. Expression pattern and mRNA abundance of growth hormone receptor (GHR) in channel catfish tissues as determined by real-time quantitative PCR. GHR copy number was normalized as a ratio to total RNA. Values represent means + S.E.; $N = 4$ of four individual catfish. Significant differences ($P < 0.05$) in tissue mRNA levels are indicated by different letters.

3.1.3. Gene expression and tissue distribution

Real-time PCR indicated that GHR was predominantly expressed in the liver of channel catfish (Fig. 3). Substantial levels of mRNA were also detected in the gall bladder, gill, heart, and spleen. GHR mRNA was detected in all tissues analyzed, with the lowest levels being detected in the anterior-intestine and hypothalamus.

3.2. Effects of food deprivation and cortisol administration

3.2.1. Weight gain

On average, catfish in the fed-control treatment (fed daily to apparent satiation) demonstrated a 156% increase in weight, gaining 47.9 g during the 4-week feeding study (Table 2).

Table 2
Weight gain and plasma IGF-I concentrations of channel catfish after 4 weeks of satiation feeding ("fed-control"), food deprivation ("fasted"), or dietary cortisol administration ("cortisol-fed"; 200 mg/kg feed)

Treatment	Weight gain (g)	Plasma IGF-I (ng/mL)
Fed-control	47.9 ± 4.7^{a}	4.6 ± 1.1^{a}
Fasted	-5.1 ± 1.4^{c}	1.0 ± 0.03^{b}
Cortisol-fed	8.6 ± 2.9^{b}	1.0 ± 0.02^{b}

Means ± S.E.; $N = 3$ within a column having different superscripts (a–c) are significantly different ($P < 0.05$).

Food deprivation for 4 weeks resulted in 18% weight loss in the fasted treatment. Exogenous cortisol administration significantly depressed weight gain, with cortisol-fed fish gaining only 8.6 g (28%) after 4 weeks.

3.2.2. Plasma IGF-I concentrations

A significant reduction ($P < 0.05$) in plasma IGF-I concentrations was observed in the fasted and cortisol-fed treatment groups compared to the fed-control treatment group (Table 2). Circulating IGF-I concentrations were 4.6 times higher in the fish fed daily to apparent satiation. Plasma IGF-I concentrations were positively correlated to hepatic GHR mRNA expression ($r = 0.55$, $P < 0.05$).

3.2.3. Hepatic GHR expression

Results of real-time PCR demonstrated a significant reduction ($P < 0.05$) in hepatic GHR expression when catfish were food-deprived for 4 weeks compared to the fed-control treatment group. A further reduction ($P < 0.05$) of GHR abundance was observed in the livers of catfish fed exogenous cortisol for the same period of time (Fig. 4). On average, hepatic GHR mRNA copy number was 2.8 times lower in fasted fish, and 9.4 times lower in cortisol-fed fish.

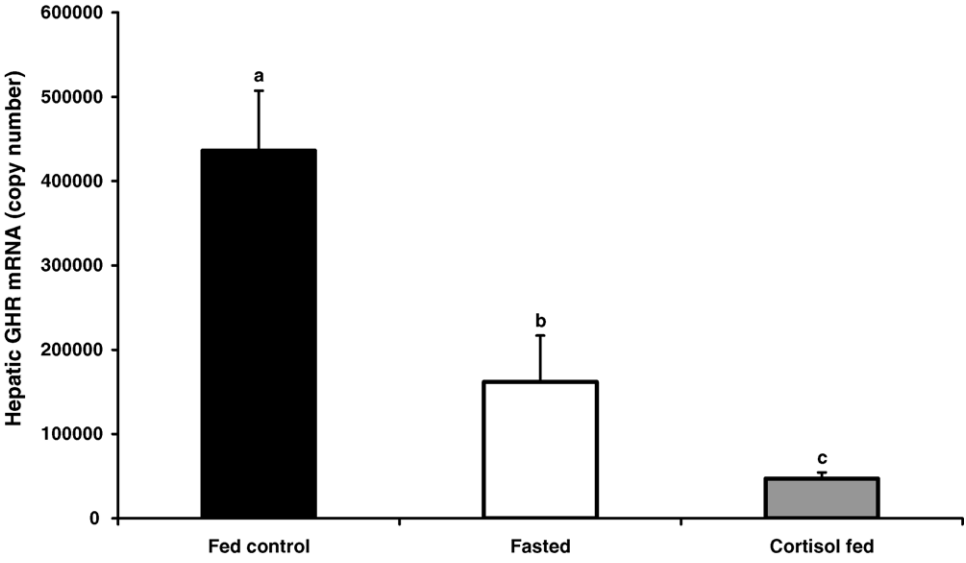

Fig. 4. Differences in channel catfish hepatic growth hormone receptor (GHR) mRNA levels after 4 weeks of satiation feeding ("fed-control"), food deprivation ("fasted"), or dietary cortisol administration ("cortisol-fed"; 200 mg/kg feed). GHR mRNA copy number was normalized against 18S ribosomal RNA. Values represent means + S.E. of three tanks ($N = 3$). Two catfish per tank were individually analyzed and averaged by tank. Significant differences ($P < 0.05$) in mRNA levels among treatments are indicated by different letters.

4. Discussion

The present study was dependent upon identifying and sequencing the channel catfish growth hormone receptor gene, which we have characterized based on genomic DNA and hepatic cDNA sequence. Channel catfish GHR contains numerous features conserved among vertebrate GHRs and many similarities with salmonid GHRs that support the correct identification of the gene. Among the conserved characteristics are five cysteine residues in the extracelluar domain (Fig. 1). Prior to the characterization of salmon GHR cDNA sequences is was thought that all vertebrate GHRs had seven conserved extracellular cysteine residues. In other vertebrates, six cysteine residues are involved in disulfide bond formations within the receptor while the seventh remains unpaired prior to GH binding [29]. It has been suggested that the lack of a third disulfide bond in salmonids might result in a less constrained structure for the extracellular region [30]. A similar conclusion might be suggested for channel catfish GHR, but the significance of a potentially less constrained structure in the extracellular region of these fish GHRs has yet to be elucidated. All vertebrate GHRs examined to date, including the channel catfish GHR, also have an unpaired cysteine residue in the extracellular domain, which is important for GH-induced receptor disulfide linkage [31].

Four potential *N*-glycosylation sites also exist in the extracellular domain of the channel catfish GHR (Fig. 1). The first two sites (aa 30 and 58) have been observed in several other vertebrates, including goldfish, pigeon, and rat. The third site, located at amino acid 99 is conserved in all non-fish vertebrates, as well as goldfish, carp, and salmonids. The last extracellular *N*-glycosylation site (aa 139) is conserved in all known vertebrate GHRs. Further examination of the channel catfish GHR sequence also revealed a single transmembrane domain characteristic of all cytokine receptors, conserved cytoplasmic Box 1 and Box 2 regions, and conserved intracellular tyrosines (Fig. 1). Phylogenetic analysis (Fig. 2) based upon the alignment of amino acids of known growth hormone receptors and trout prolactin receptor resulted in the channel catfish GHR grouping more closely to salmond GHRs than any other receptor group. Furthermore, the gene expression results presented here also suggest conservation of function, with GHR mRNA abundance being greatest in the liver (Fig. 3) and demonstrating a classic vertebrate response to fasting (Fig. 4).

After 4 weeks of fasting in the present study, expression of hepatic GHR mRNA in channel catfish was significantly depressed, having 2.8 times lower copy number than fed-control fish. Fukada et al. [26] also reported a reduction in hepatic GHR mRNA in masu salmon after prolonged fasting. These two studies demonstrate a conserved function of GHR during fasting in fish that has long been suspected, that of GHR down-regulation and loss of hepatic GH sensitivity. In other vertebrates, hepatic GHR receptors have been observed to diminish in number during fasting, presumably a result of decreased mRNA reducing receptor synthesis. This reduction in GHR receptor expression has been associated with hepatic growth hormone resistance in many vertebrates and results in reduced circulating IGF-I. For a review of growth hormone resistance, see Harvey et al. [25].

The literature contains many examples of alterations in circulating IGF-I and GH during periods of fasting in fish [32–36], channel catfish included [11,12,18]. After 4 weeks of fasting, Small and Peterson [19] reported both an increase in circulating GH and a decrease in plasma IGF-I levels. In other teleost fishes, reduced hepatic binding capacity for GH during

fasting appears to be one of the mechanisms responsible for the decline in circulating IGF-I [33,37,38]. Small and Peterson [19] hypothesized that the observed increase in channel catfish plasma GH after 4 weeks of fasting was due to low circulating IGF-I, resulting in reduced negative feedback on GH synthesis and release. This is supported by observations in rainbow trout, where IGF-I has been shown to negatively regulate GH release [39]. The present study further supports this mechanism by demonstrating a reduction in hepatic GHR expression correlated to reduced plasma IGF-I.

Prior to the present study, the effect of cortisol on GHR mRNA expression in fish had not been reported, yet there was reason to suspect an interaction between the two. In gobies, plasma cortisol levels have been reported to increase significantly during fasting [40], and Peterson and Small [11] reported an increase in plasma cortisol levels of channel catfish fasted for 30 days. The effect of fasting in catfish was less clear than in gobies, with no observed effect of fasting on plasma cortisol levels after 14, 45, and 60 days of fasting [11]. Nonetheless, the potential for increased levels of circulating cortisol during fasting is intriguing when considering growth is also depressed during periods of prolonged stress, and in rats glucocorticoids are required for increases in both protein degradation and levels of mRNAs encoding components of the ubiquitin-proteasome proteolytic system during catabolic conditions causing muscle atrophy [41,42].

Administration of exogenous cortisol to channel catfish has been previously reported to reduce weight gain, feed intake, and plasma IGF-I levels [18]. The present study verifies those results and demonstrates reduced hepatic GHR expression in catfish fed cortisol for 4 weeks. Feeding cortisol had a greater effect on reducing GHR expression than did fasting. One possible explanation might be differences in plasma cortisol profiles over the entire 4 weeks. The response of cortisol to fasting appears to be dynamic, possibly responding to changes in metabolic status [11], but by feeding cortisol in the present study plasma levels were likely elevated throughout the entire 4 weeks, as previously demonstrated [18]. Greater reduction of hepatic receptor expression by cortisol relative to fasting did not result in further reduction of plasma IGF-I levels or fish weight. Fasted fish lost weight while cortisol-fed fish did not (exhibiting reduced growth), implying other mechanisms, such as nutrient deficiencies, are involved in fasting-induced weight loss.

It has been known for some time that cortisol administration reduces somatic growth in fish [4,5]; however, the mechanisms of cortisol-induced growth inhibition have received little attention. In mammals, increased glucocorticoid levels, whether endogenous or exogenous, have long been associated with stunted growth [43] and muscle atrophy [44]. It has been suggested that glucocorticoids may retard growth by directly altering GH secretion or by antagonizing GH action [45]. Furthermore, glucocorticoids are required for the activation of the ubiquitin-proteasome proteolytic system in rats with catabolic conditions causing muscle atrophy. In humans, glucocorticoids appear to reduce pituitary GH responsiveness to a variety of secretagogues [46]. Dexamethasone has been shown to inhibit the GH-induced increases in hepatic IGF-I mRNA of hypophysectomized rats, and GH fails to reverse glucocorticoid-induced reduction of serum IGF-I in normal rats [47]. Furthermore, Beauloye et al. [45] observed a parallel decrease in GHR mRNA and GH-induced IGF-I mRNA in rat primary cultured hepatocytes, concluding that GH resistance caused by dexamethasone is mediated by diminished GH receptor synthesis.

In conclusion, we have identified, sequenced, and characterized the growth hormone receptor gene from channel catfish, and utilized the sequence information to develop a real-time PCR assay for measuring hepatic GHR expression in control-fed, fasted, and cortisol-fed catfish. Results of this study demonstrate a classic fasting-induced reduction in hepatic GHR expression and concurrent reductions in growth and circulating IGF-I in channel catfish. Administration of dietary cortisol for 4 weeks also reduced growth, plasma IGF-I, and hepatic GHR expression, suggesting that situations resulting in elevated circulating cortisol levels, such as stress and fasting, may result in diminished growth hormone receptor synthesis and hepatic growth hormone resistance. Further research is needed to better characterize the interactions between the somatotropic and corticotropic axes in fish.

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